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# MYOFIBRILLAR FATIGUE *VERSUS* FAILURE OF ACTIVATION DURING REPETITIVE STIMULATION OF FROG MUSCLE FIBRES

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SUMMARY

1. Single fibres isolated from the anterior tibialis muscle of *Rana temporaria* (temperature, 2–5 °C; sarcomere length, 2·10 μm) were fatigued using two separate protocols that led to different degrees of depression of tetanic force. Under control conditions the fibre was stimulated to produce a 1 s fused isometric tetanus at 300 s intervals. A moderate degree of fatigue (tetanic force reduced to 70–80% of the control value) was produced by decreasing the intervals between tetani to 15 s ('fatiguing protocol 1'). A more pronounced depression of tetanic force (to 40–50% of the control value) was produced by evoking a single twitch at 1–2 s intervals ('fatiguing protocol 2').

2. Fatiguing protocol 1 reduced the contracture response to submaximal and supramaximal concentrations of caffeine (3–15 mM) in proportion to the decrease in tetanic force. These results support the view that fatiguing stimulation according to protocol 1 leads to a true 'myofibrillar fatigue' with no failure of activation of the muscle fibre.

3. Fatiguing protocol 2 reduced the amplitudes of isometric twitch and tetanus to below 10 and 50% of the control values, respectively. By contrast, the maximal contracture response to caffeine (15 mM) was depressed by merely 2–3% of its prefatigue value.

4. Force and instantaneous fibre stiffness were recorded simultaneously during twitch and tetanus as fatigue was induced by protocol 2. During the initial part of fatigue (tetanic force reduced by 25% of control) stiffness was reduced by merely 9% in accordance with previous measurements during fatigue induced by protocol 1. However, with further depression of twitch and tetanus by protocol 2 there was a marked reduction of fibre stiffness. These results, together with the findings reported under point 3, strongly suggest that at an advanced state of fatigue induced by protocol 2 the decrease in active force is largely due to failure of activation of the contractile system.

5. Muscle fibres were quickly frozen for electron microscopical examination after shortening below slack length (to approximately 1·6 μm sarcomere spacing) during tetanic stimulation. In non-fatigued fibres, and in fibres fatigued according to protocol 1, the myofibrils exhibited a straight appearance throughout the preparation suggesting that the entire volume of the fibre was properly activated. In fibres

fatigued by protocol 2, on the other hand, only the most peripheral layers of myofibrils remained straight after shortening, whereas the centre of the fibre showed marked waviness indicating failure of the inward spread of activation in this case.

## INTRODUCTION

The contractile strength of isolated skeletal muscle is temporarily reduced after a period of sustained or repetitive mechanical activity. This reversible decrease in contractile performance, generally referred to as 'fatigue', has been explored in numerous previous studies of isolated muscle preparations (e.g. Edwards, Hill & Jones, 1975; Dawson, Gadian & Wilkie, 1978, 1980; Edman & Mattiazzi, 1981; Lännergren & Westerblad, 1986, 1989, 1991; Cooke, Franks, Luciani & Pate, 1988; Allen, Lee & Westerblad, 1989; Curtin & Edman, 1989, 1991; Edman & Lou, 1990). As first demonstrated in frog single muscle fibres (Edman & Mattiazzi, 1981), fatigue does not merely reduce the muscle's capacity to produce force; it also diminishes the muscle's ability to produce motion, manifesting itself as a decrease in the speed of shortening at zero load (also see Lännergren & Westerblad, 1989; de Haan, Jones & Sargeant, 1989; Curtin & Edman, 1991). The experimental evidence suggests that the reduced mechanical performance at a moderate degree of fatigue is attributable to altered kinetics of cross-bridge function without any appreciable change in the state of activation of the contractile system (Edman & Lou, 1990). In accordance with this view the tetanic output of a muscle fibre in moderate fatigue (tetanic force reduced to 70–75 % of the rested-state value) is not enhanced by caffeine, indicating that the contractile system is fully activated in the fatigued state (Edman & Lou, 1990). The fact that instantaneous stiffness is only slightly reduced in a moderately fatigued muscle fibre (Edman & Lou, 1990) supports the view that the decrease in active force is only partly due to fewer active cross-bridges. The contractile failure of the muscle after fatigue is therefore mainly attributable to reduced force output of the individual bridge.

In apparent conflict with the above statements, several previous investigations have led to the conclusion that failure of activation of the contractile system is the main cause of the reduced mechanical performance during muscle fatigue. Reduction of the intracellular calcium transient during twitch and tetanus, failure of the inward spread of activation into the muscle fibre and lack of influence of fatiguing stimulation upon the contracture response to caffeine are observations that have been put forward to support this view (Eberstein & Sandow, 1963; Nassar-Gentina, Passonneau & Rapoport, 1981; Kanaya, Takauji & Nagai, 1983; Lännergren & Westerblad, 1989; Allen *et al.* 1989; Jones & Sacco, 1989; Garcia, Gonzalez-Serratos, Morgan, Perreault & Rozycka, 1991). These studies all used an intense stimulation programme that left very little time, generally no more than 1–3 s, for recovery of the muscle between the stimulation volleys. The short intervals between stimuli may be presumed to cause accumulation of potassium in the transverse tubules (e.g. Hnik, Holas, Krekule, Kriz, Mejsnar, Smiesko, Ujec & Vyskocil, 1976; Juel, 1986) with consequent depolarization of the T-tubular wall and failure of propagation of the action potential along the T-tubules towards the centre of the fibre. Thus, by increasing the contraction frequency above a certain level, in an attempt to further strengthen the fatiguing effect, the paradoxical situation may arise that a

progressively smaller volume of the fibre becomes activated while a correspondingly larger portion of the myofibrillar mass stays inactive and therefore resting.

The present study has been undertaken to further elucidate the decrease in contractile performance during fatiguing stimulation of isolated skeletal muscle. The experiments have been carried out on single muscle fibres of the frog, and the fatiguing conditions have been selected to make it possible to explore, separately, the two categories of 'fatigue' discussed above, i.e. the 'myofibrillar fatigue' that results from excessive mechanical activity *per se* and the decrease in contractile strength that is due to failure of activation of the muscle fibre. Some of the results have been presented in a preliminary form (Edman & Lou, 1989; Lou & Edman, 1990).

## METHODS

*Preparation and mounting.* Single fibres were isolated from anterior tibialis muscles of cold-adapted *Rana temporaria* as previously described (Edman, 1979). The frogs were killed by decapitation followed by destruction of the spinal cord. The fibre was mounted horizontally in a temperature-controlled Perspex chamber between a force transducer and an arm extending from an electromagnetic puller. In general both tendons were held by clips of aluminium foil as described earlier (Edman & Reggiani, 1984). In experiments where stiffness was recorded one of the tendons was instead tied by nylon threads to the distal end of the puller arm. Firm attachment of the tendon was ensured by winding two layers of Parafilm around the tendon on the puller arm (Edman & Lou, 1990).

Cross-sectional area, fibre length and sarcomere length (laser diffraction) were determined as described previously (Edman & Reggiani, 1984).

*Muscle chamber.* The muscle chamber generally used allowed quick exchanges of solution appropriate for studying potassium- or caffeine-induced contractures. A detailed description of this chamber has been given earlier (Andersson & Edman, 1974). Information concerning bath temperature and flow rate is given under 'Solutions'. A second muscle chamber was used for stiffness measurements. The latter chamber was provided with a slit in one of its short sides to enable passage of the horizontal arm of the fast electromagnetic puller that was used in these experiments (see Edman & Lou, 1990). Both chambers were provided with a jacket for circulation of a thermostat-controlled water–glycol mixture.

*Force transducer and electromagnetic pullers.* The force transducer used for standard measurements was of the same design as that described by Edman & Reggiani (1984). The force transducer and puller employed for stiffness measurements have previously been described (Edman & Lou, 1990).

*Solutions.* The standard Ringer solution had the following composition (mM): NaCl, 115·5; KCl, 2·0; $CaCl_2$, 1·8; $Na_2HPO_4 + NaH_2PO_4$, 2·0; pH, 7·0. Caffeine-Ringer solution: standard Ringer solution plus caffeine in concentrations ranging from 3 to 15 mM. High-potassium Ringer solution: KCl, 117·5; $CaCl_2$, 1·8; $Na_2HPO_4 + NaH_2PO_4$, 2·0; pH, 7·0. All solutions were precooled before entering the muscle chamber. The standard Ringer solution was perfused through the chamber (volume *ca* 2·5 ml) at a speed of approximately 2 ml/min. The caffeine-Ringer and the high-potassium Ringer solutions were flushed through the muscle chamber at a rate of approximately 3 ml/s providing a virtually complete exchange of solution within 3 s (see further Andersson & Edman, 1974). The bath temperature varied between 2·0 and 4·8 °C in the different experiments but was constant to within ±0·2 °C during any given experiment. During contractures induced by precooled caffeine or high-potassium Ringer solution (see above) the bath temperature was reduced to 0·1–0·5 °C.

*Stimulation.* For stimulation, rectangular pulses of 0·2 ms duration were delivered between two platinum plate electrodes placed symmetrically on either side of the preparation approximately 2 mm from it. The stimulus strength was approximately 15% above threshold. Isometric (fixed fibre ends) tetani of 1–10 s duration were produced at regular intervals (see later) by a train of pulses of appropriate frequency (15–22 Hz). The pulse frequency was just sufficient to provide complete mechanical fusion under the conditions used.

*Stiffness measurement.* Fibre stiffness was measured as the change in force that occurred in response to a fast, low-amplitude length perturbation of the muscle fibre using the technique described by Edman & Lou (1990). A 4 kHz sinusoidal length oscillation of constant amplitude was

applied to one end of the fibre and the corresponding change in force was recorded. The peak-to-peak amplitude of the length oscillation was 10–11 $\mu$m corresponding to 1·4–1·8 nm/half-sarcomere in the different experiments. The length oscillation was applied throughout a contraction period. The signals from the displacement and force transducers were passed through narrow bandpass filters and precision rectifier circuits in this way providing a continuous measurement of the peak-to-peak amplitude of the oscillatory length change and of the resulting change in force (for further details, see Edman & Lou, 1990).

*Contractures induced by caffeine and high potassium concentration.* In some experiments, to be described separately, contractures induced by caffeine or high potassium concentration were interposed at intervals of 40–60 min between tetanic contractions. These relatively long intervals between contractures were used to allow time for complete recovery of the tetanus responses after a preceding caffeine or potassium contracture. Using this approach there was generally no sign of deterioration of the contractile strength after several hours of experimentation. Before inducing a contracture it was ascertained that the caffeine or potassium solution to be flushed into the muscle chamber had a temperature of 0·1–0·5 °C.

*Experimental procedure.* The fibres were generally mounted in the test chamber for 1–2 h before the experiment was started and were tetanized occasionally during this time. The resting sarcomere length was set to 2·1 $\mu$m. During the actual experiment the fibre was stimulated to produce a 1 s isometric tetanus at regular 5 min intervals until constant responses were obtained. This initial *control period* lasted for at least 30 min. Two different protocols were used for producing fatigue. *Fatiguing protocol 1:* the fibre was stimulated to produce a 1 s tetanus at 15 s intervals. By this stimulation programme the tetanic force was reduced to a steady level, approximately 25% below the control value, within 10 min (Edman & Mattiazzi, 1981; Edman & Lou, 1990). *Fatiguing protocol 2:* the fibre was stimulated to produce a twitch at 1–2 s intervals allowing the fibre to fully relax between stimuli. By this procedure peak twitch force was reduced to approximately 10% of the control value in 13–16 min. During the fatiguing process a 1 s isometric tetanus was induced in place of the twitch every 5 min.

*Recording and measurement of responses.* Force and length signals were displayed and photographed on a Tektronix 5103N storage oscilloscope or recorded on a Nicolet 4094B digital oscilloscope. The film records were measured at 10 × magnification in a Nikon model 6C profile projector using the stage micrometer readings (Edman, 1979).

Student's *t* test was used for determinations of statistical significance. All statistics are given as means ± S.E.M.

*Electron microscopy.* A special set-up was used for rapid freeze fixation of the muscle fibre during contractile activity. The fibre was mounted horizontally between a force transducer and an electromagnetic puller as described above. Both force transducer and puller were secured on individual stands and were not attached to the muscle chamber. The latter was made of plexiglass and was constantly perfused with temperature-controlled Ringer solution (see above). The chamber could be slid vertically along a rack between two extreme positions leaving the fibre either submerged in Ringer solution (chamber in upper position) or suspended in air approximately 10 mm above the fluid level (chamber in lower position). In either of these situations the fibre could be stimulated to produce a single twitch or a 1 s fused tetanus by passing current between the aluminium foils that were attached to the tendons at each end of the fibre. The stimulus strength was adjusted appropriately to be 10–20% above the threshold value in both cases. The fibre was allowed to stay in air for at most 10 s at a time. Such exposures to air could be repeated at 2 min intervals over hours without noticeable deterioration of the fibre. When required (see below) the chamber could be rapidly elevated by actuating a piston (driven by compressed air) that governed the vertical movement of the chamber. The time taken for a fibre of 100 $\mu$m thickness to pass through the air–fluid interface during this manoeuvre was less than 1 ms.

For rapid freeze fixation of the fibre the plexiglass chamber was exchanged for a thermally insulated trough that was filled with liquid nitrogen and that had a central compartment containing the freezing medium, a propane–propylene mixture. The trough could be rapidly elevated (see above) to submerge the fibre in the freezing medium at a pre-set time during stimulation. Generally the fibre remained intact at the end of this procedure but sometimes had a fracture near one of the tendon insertions.

The temperature measured close to the fibre when suspended in air (approximately 24 °C) was only slightly reduced (by less than 1 °C) when the freezing medium was put in place below the fibre.

However, the temperature inside the fibre can be presumed to be lower than that of the surrounding air due to evaporation of water from the fibre surface. The actual temperature inside the fibre was estimated from the change in fusion frequency that occurred when the preparation was moved from the Ringer solution (3–5 °C) and was placed in air above the freezing medium. The measured change of this mechanical parameter corresponded to a difference in temperature of approximately 5 °C. The temperature of the fibre during the final tetanus (during which freeze fixation was performed) was thus estimated to be 8–10 °C in the present series of experiments.

After freezing, the fibre was transferred from the propane–propylene medium into liquid nitrogen. Freeze substitution was carried out in acetone, to which 5% osmic acid had been added, using an approach similar to that described by Padron, Alamo, Craig & Caputo (1988). The temperature was thereby steadily increased from −193 to −20 °C within approximately 45 h. After freeze substitution the preparation was stained for 2 h in 5% uranyl acetate dissolved in ethanol. The fibre was finally divided into segments, approximately 1 mm in length, which were embedded in Epon. Longitudinal sections passing through the centre of the fibre were sampled for electron microscopical examination. The direction of sectioning was such that the knife edge was parallel with the long axis of the fibre. The sections were stained in 4% uranyl acetate and lead citrate (Reynolds, 1963) and were examined in Jeol 100 and Jeol 200 electron microscopes.

*Procedure of freezing fibre during fatigue*

After mounting the fibre between the force transducer and puller the sarcomere length was adjusted to 2·10 μm and the appropriate stimulation strength and pulse frequency required for tetanization of the fibre in air and Ringer solution were determined (see above). The fibre was generally kept mounted in the experimental chamber for several hours before the actual experiment was started and was tetanized occasionally in air and in Ringer solution during this time.

The experiment started with a control period during which the fibre, immersed in Ringer solution at 3–5 °C, was stimulated to produce a 1 s fused tetanus at 5 min intervals. After constant tetanic responses had been attained, fatigue was initiated by switching to either of the two fatiguing protocols described above. When a steady fatigue level was reached (for further details see Results), the stimulation was stopped and the muscle chamber was lowered from the fibre. After the trough containing the freezing medium had been put into place, the fibre was stimulated to produce a final 1 s tetanus while suspended in air. At a given time during the plateau phase of this tetanus the container with freezing medium was suddenly raised to submerge the fibre in the propane–propylene (−193 °C) mixture. The time between the last stimulus in Ringer solution and freeze fixation did not exceed 20 s. During this interval the fibre was exposed to air for no more than 10 s (see earlier). The same procedure was used for freeze fixation of non-fatigued fibres. In this case the final tetanus (during which freezing was performed) was timed to occur 5 min after the nearest preceding stimulation period.

## RESULTS

### *Effects of fatigue on isometric tetanus and contracture responses to caffeine and high potassium concentration*

In the present series of experiments the effects of fatiguing stimulation on the isometric tetanus were correlated with concurrent changes of the contracture response to caffeine or high potassium concentration. Two different fatiguing protocols were used (see Methods), one which produced a moderate degree of fatigue (protocol 1) and another more intense stimulation programme which led to severe depression of both isometric twitch and tetanus (protocol 2).

### *Fatigue induced according to protocol 1*

Figure 1 illustrates concomitant changes in maximum tetanic force and caffeine-induced contracture during development of fatigue. During an initial control period, covering approximately 2–4 h, the fibre was stimulated to produce an isometric

tetanus at regular 5 min intervals. On two occasions during this period a contracture was initiated (in place of a tetanus) by rapidly exposing the fibre to a Ringer solution containing 3 or 15 mM caffeine. The latter caffeine concentration was sufficient to produce a maximal contracture response under the experimental conditions used (see

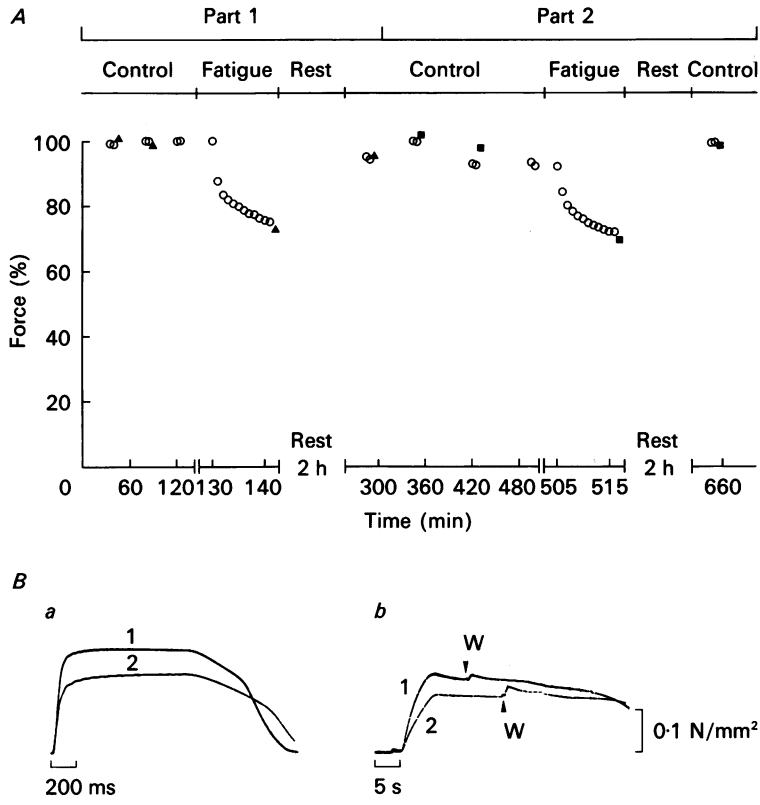


Fig. 1. *A*, relative changes of tetanic force and contracture responses to caffeine during development of fatigue in a single muscle fibre subjected to fatiguing protocol 1. ○, isometric tetanus; ▲ and ■, contracture responses to 15 and 3 mM caffeine, respectively. The sequence of control, fatigue and rest periods is indicated by marked intervals above the data points. The time scale is expanded during the fatigue period. Only some of the tetanic responses are plotted in the diagram for clarity. Tetanic tensions throughout the experiment are normalized to the mean value of tetanic force derived during the control period of part 1. Contracture tensions being induced by two different caffeine concentrations are normalized to values derived during the control period in each respective part of the experiment. Note that tetani and caffeine contractures are depressed to approximately the same degree. *B*, superimposed records of *a*, tetanic force and *b*, caffeine (15 mM) contracture under control conditions (traces 1) and after fatiguing stimulation (traces 2). Arrow-heads (W) indicate time where contracture solution is replaced by ordinary Ringer solution. Transient increase in force is attributable to the somewhat higher temperature of the incoming washing solution. Same fibre as in *A*. Temperature, 3·3 °C.

Fig. 2, inset). In agreement with earlier results (Lännergren & Westerblad, 1989), maximum force attained during the caffeine contracture was *ca* 80 % of that reached during tetanus (cf. control myograms of tetanus and caffeine contracture in Fig. 1*B*).

Fatigue was produced by reducing the intervals between tetani to 15 s. This stimulation protocol led to a continuous decrease in maximum tetanic tension with the force levelling off at approximately 75 % of the control value within 10 min at the high stimulation frequency. In accordance with earlier results (Edman & Mattiazzi,

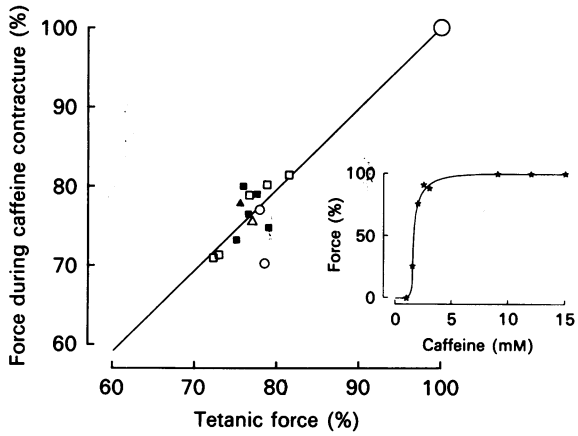


Fig. 2. Relation between maximum tetanic force and maximum contracture response to caffeine during development of fatigue induced by protocol 1. Data expressed as percentage of tetanus and contracture tensions recorded under control (prefatigue) conditions in respective fibres. The control value (indicated by large open circle) is the mean of two to four separate recordings in each fibre. Caffeine concentrations (mM): □, 3; ○, 6; △, 9; ▲, 12; ■, 15. Continuous line, regression of caffeine contracture ($F_{con}$) upon tetanic force ($F_{tet}$) based on all data points: $F_{con} = 1{\cdot}02\,F_{tet} - 2{\cdot}38$ (correlation coefficient, 0·99; $P < 0{\cdot}001$; $n = 28$). Inset: relation between peak contracture tension and caffeine concentration. Each data point is the mean of two to nine contractures performed in altogether nine fibres. Force values are normalized to the maximum contracture response derived by either 12 or 15 mM caffeine in each respective fibre.

1981) fatiguing stimulation also reduced the rate of rise of force and markedly prolonged the relaxation phase of the isometric tetanus (Fig. 1*B*). As is illustrated in Fig. 1*A* and *B*, the contracture response to caffeine (3 and 15 mM) was reduced to roughly the same degree as the tetanic tension was by fatiguing stimulation. After resting the fibre for approximately 2 h in ordinary Ringer solution both tetanus response and caffeine contracture were restored to their prefatigue levels.

Ten experiments similar to that illustrated in Fig. 1 were performed in which the caffeine concentration used for contractile activation varied between 3, 6, 9, 12 and 15 mM. In several of these experiments two fatigue periods, separated by a 2 h rest interval, were produced in the same fibre. As can be seen from the inset of Fig. 2, maximum contracture response was attained with 12–15 mM caffeine. At 3 mM caffeine concentration contracture tension was approximately 90 % of maximum. The results shown in Figs 1 and 2 clearly demonstrate, however, that, irrespective of the caffeine concentration used, fatiguing stimulation depressed the amplitude of the caffeine contracture to nearly the same degree as it depressed the tetanic force.

In three experiments the effects of fatigue were compared during isometric tetanus and potassium-induced contracture using the same experimental procedure as

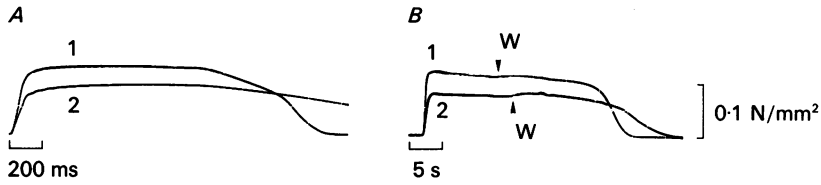


Fig. 3. Superimposed records of tetanic force (*A*) and contracture response to 117·5 mM potassium (*B*) derived from a single muscle fibre under control conditions (traces 1) and after fatiguing stimulation according to protocol 1 (traces 2). Note that fatiguing stimulation leads to approximately the same degree of force depression during tetanus and potassium-induced contracture. Arrow-heads (W) indicate time where contracture solution is replaced by ordinary Ringer solution. Temperature, 3·4 °C.

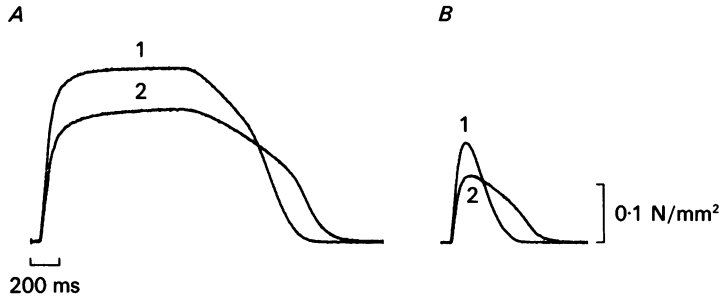


Fig. 4. Superimposed records of isometric tetani (*A*) and twitches (*B*) under control conditions (traces 1) and after fatiguing stimulation according to protocol 1 (traces 2). Temperature, 2·3 °C.

described above for caffeine. Oscilloscope records from one experiment are illustrated in Fig. 3. The peak force recorded during potassium contracture under control (prefatigue) conditions can be seen to be quite close to maximum tetanic force (cf. myograms 1 in Fig. 3). In the three experiments performed in this series the peak amplitude of the potassium contracture was $95{\cdot}4 \pm 0{\cdot}9$ (S.E.M.) % of the tetanic tension during the control period. Similar to the situation during caffeine contractures (see earlier), fatiguing stimulation led to a reduction of the contracture response to potassium that was proportional to the decrease in tetanic force. A mean reduction of tetanic tension to $75{\cdot}3 \pm 2{\cdot}6$ (S.E.M., $n = 3$) % during fatigue was thus found to be associated with a decrease to $69{\cdot}0 \pm 3{\cdot}2$ % of the peak contracture force to high potassium concentration.

With the above fatiguing protocol (1 s isometric tetanus induced at 15 s intervals) the twitch response was only slightly more depressed than was the isometric tetanus (cf. fatigue produced according to protocol 2 below). In the experiment illustrated in Fig. 4, the peak twitch force was reduced to 67 % of the control value by fatiguing stimulation as compared to a decrease to 76 % of the isometric tetanus. Similar to the situation during tetanus the rate of rise of force during the onset of the twitch and the rate of force decline during twitch relaxation were both markedly reduced in the fatigued state.

*Fatigue induced according to protocol 2*

In the following experiments a fatiguing protocol was employed that affected contractile performance more extensively than did that described in the preceding section. During an initial control period of 2–4 h single muscle fibres were stimulated

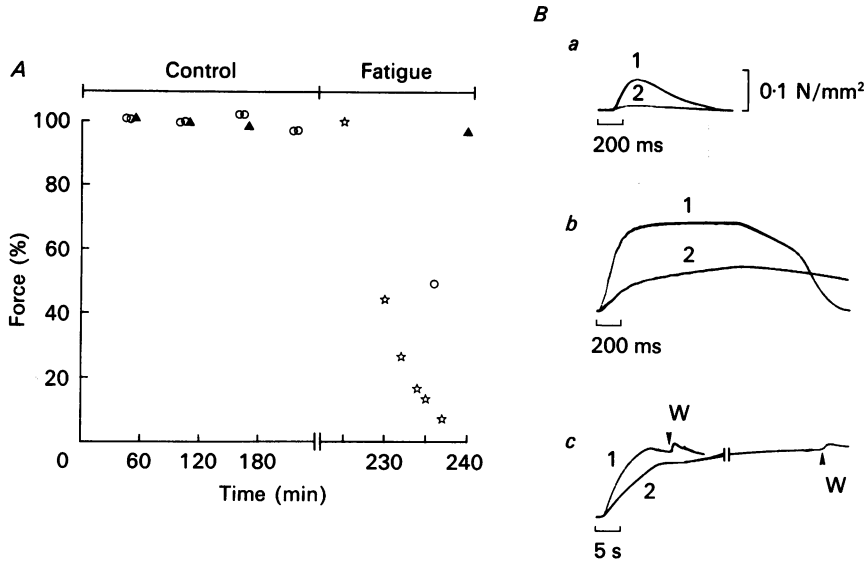


Fig. 5. *A*, relative changes of tetanic force (○), isometric twitch (☆) and contracture response to 15 mM caffeine (▲) in a single muscle fibre subjected to fatiguing stimulation according to protocol 2. Tensions during tetani, twitches and caffeine contractures are normalized in each case to the mean values derived during the control (prefatigue) period. Marked intervals above the data points indicate time span of control and fatigue periods. The time scale is expanded during fatigue. *B*, sample records from a single muscle fibre (same as in *A*) illustrating isometric twitch (*a*), tetanus (*b*) and contracture response to 15 mM caffeine (*c*) under control conditions (traces 1) and after development of fatigue according to protocol 2 (traces 2). Note that fatiguing protocol 2 leads to extreme depression of the isometric twitch, marked reduction of the tetanic force but no significant change of the total amplitude of the caffeine contracture. There is pronounced tension creep during both tetanus and caffeine contracture in the fatigued state. Arrow-heads (W) in *c* indicate time when caffeine solution is replaced by ordinary Ringer solution. Trace 2 in *c* is composed of two oscilloscope sweeps separated by approximately 2 s. Temperature, 3·4 °C.

to produce a 1 s isometric tetanus at 5 min intervals. Two or three contractures to 15 mM caffeine were also performed during this initial period at intervals of 45–60 min (for further details, see Methods). Forty-five to sixty minutes after the last contracture, when consistent tetanic responses had been attained, fatigue was initiated by stimulating the fibre to produce an isometric twitch at 1–2 s intervals.

Results from a representative experiment are illustrated in Fig. 5. The fatiguing protocol used can be seen to cause a very marked reduction of the isometric twitch, the peak twitch force being depressed to below 10% of its control value in approximately 15 min from the end of the control period. The tetanus response was also greatly reduced as the fibre was fatigued by using protocol 2 but less so than was

the isometric twitch. In eleven experiments in which the twitch amplitude was depressed to 5·0–9·8% of its control value the tetanic force produced during 1 s stimulation was 46·3 ± 3·0% (± S.E.M.) of the prefatigue value. It is of interest to note that at this point of fatiguing stimulation the fibre was capable of producing a nearly maximal (97–98%) contracture response to 15 mM caffeine (Fig. 5*A* and *B*). The rate of rise of force during the caffeine contracture was, however, markedly reduced in the fatigued state. Thus whereas maximum force was attained within 5–10 s under control conditions, a time period of nearly 1 min was required to reach maximum contracture force in fatigue. The results presented in Fig. 5 suggest that fatiguing protocol 2 leads to a failure of the excitation–contraction coupling that is circumvented as the fibre is activated by a supramaximal concentration of caffeine (see Discussion).

The tetanus response exhibited a substantial amount of tension creep after the fibre had been fatigued according to protocol 2. Thus as can be seen in Fig. 5*B* tetanic tension continued to climb slowly after the initial, steep rise of force. This is noteworthy in view of the fact that tension creep due to sarcomere non-uniformity is negligible at the sarcomere length (2·1 $\mu$m) considered (Edman & Reggiani, 1984). As an attempt to further characterize the tension creep, seven experiments were performed in which the fibre, after development of fatigue, was tetanized for 2–10 s. Tension creep was found to proceed for 2–3 s after the onset of stimulation. The force reached after 2 s tetanization, i.e. at a point where tension creep had nearly come to an end, was 60·5 ± 3·0% (± S.E.M., $n = 7$) of the control (prefatigue) value.

In the following series of experiments changes in fibre stiffness and active force were correlated during fatiguing stimulation according to protocol 2. Stiffness was measured as the force response to a 4 kHz length oscillation (amplitude, *ca* 1·6 nm/half-sarcomere) that was applied to one end of the fibre during activity. With the approach used it was possible to derive a direct read-out of the stiffness, in parallel with the isometric force signal, throughout the contraction period (see Methods). Results from three such experiments are shown in Fig. 6. The initial decrease in twitch force during the fatiguing process was associated with a relatively small decline in fibre stiffness (Fig. 6*A*). Thus for a 25% decrease of peak twitch force there was merely 10–15% reduction of fibre stiffness. However, with further depression of the isometric twitch the measured stiffness was progressively reduced reaching values below 20% of the control at the end of the fatiguing period.

The relative changes in force and stiffness measured during tetanus were similar to those observed during the twitch (Fig. 6*B*). The uppermost portion of the force–stiffness relationship could not be delineated in detail since tetanic force had already declined substantially when the first tetanus was recorded during the fatiguing period. The results demonstrate, however, that the initial 25% depression of tetanic force was associated with merely 9% reduction in fibre stiffness. This value agrees well with the force–stiffness relationship that was previously recorded during fatigue induced by stimulation protocol 1 (Edman & Lou, 1990). This result supports the view (see Discussion) that fatigue down to approximately 75% of the control value involves the same basic change of the contractile process irrespective of the fatiguing protocol employed. The data in Fig. 6*B* clearly show, however, that further depression of the tetanic force by means of protocol 2 was associated with a marked

decrease in fibre stiffness. Thus, for a 60% decrease in tetanic force there was a 39% decline in stiffness. This finding suggests that reduction of the number of active cross-bridges eventually becomes a predominant cause of the force decline as the fibre is fatigued according to protocol 2.

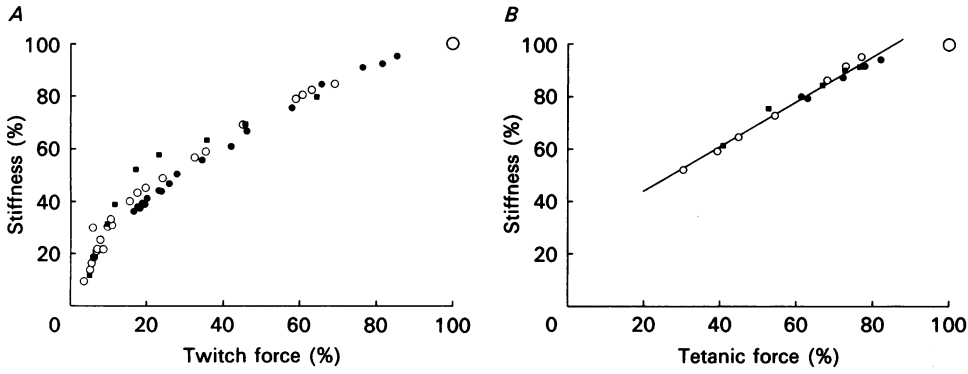


Fig. 6. *A*, relation between maximum twitch force and maximum twitch stiffness during development of fatigue in three muscle fibres subjected to fatiguing protocol 2. *B*, relation between maximum tetanic force and maximum tetanic stiffness (during 1 s stimulation) recorded in three muscle fibres (same as in *A*) during development of fatigue according to protocol 2. Data in *A* and *B* are normalized with respect to maximum force and maximum stiffness recorded under control conditions in respective fibres. The control value (indicated by large open circle) is the mean of three to four separate recordings in each of the three fibres. Data from a given fibre are denoted by the same symbol. The continuous line in *B* is the least-squares regression of stiffness ($S_{tet}$) upon force ($F_{tet}$) calculated for data points between 30 and 82% of tetanic force: $S_{tet} = 0{\cdot}86\ F_{tet} + 26{\cdot}68$ (correlation coefficient, 0·99; $P < 0{\cdot}001$; $n = 17$).

The combined force and stiffness measurement offered a possibility to further elucidate the nature of the tension creep that occurred during tetanus in the fatigued state (see earlier). Figure 7 illustrates the relationship between tetanic force and fibre stiffness during the phase of tension creep in three single muscle fibres. These measurements were carried out after the twitch force had been depressed to 9–18% of the control value in the different fibres. The results show that for each individual fibre there existed a linear relation between force and stiffness during tension creep. However, the slope of the regression between force and stiffness varied over a relatively wide range among the different fibres. The results suggest that the slow climb of force during tetanus at a high degree of fatigue is based on steady recruitment of active cross-bridges.

*Failure of the inward spread of activation during intense fatiguing stimulation*

The possibility was considered that a period of intense fatiguing stimulation might cause a progressive failure of the inward spread of the action potential along the transverse tubules (Gonzalez-Serratos, Garcia, Somlyo, Somlyo & McClellan, 1981; Garcia *et al.* 1991). This would lead to incomplete activation of the central parts of the fibre and, in the extreme case, leave the innermost portion of the fibre inaccessible for activation by the electrical stimulus. The following experiments were performed to investigate if such a failure of activation may arise in the course of

fatigue induced by stimulation protocols 1 and 2. The experiment was based on the previous observation that individual myofibrils in a fibre are unable to shorten passively below slack length (Brown, Gonzalez-Serratos & Huxley, 1984). Thus if a population of myofibrils are inactive, or only partially active, as the fibre shortens

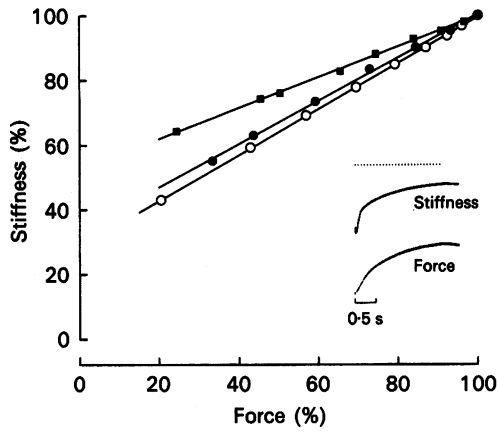


Fig. 7. Relation between force and stiffness measured during the phase of tension creep of tetanus in three muscle fibres (same fibres as in Fig. 6) that had been brought into fatigue by means of protocol 2. Data normalized with respect to the peak values of force and stiffness recorded during 2 s tetanic stimulation in the fatigued state. Data from a given fibre (denoted by the same symbol) have been fitted by a least-squares regression line. Inset, example records of force and stiffness during fused tetanus of a fatigued single muscle fibre. Note the slow climb of both force and stiffness during tetanic stimulation.

below slack length, they will assume a wavy appearance while the fully active myofibrils remain straight.

Single muscle fibres, set at a resting sarcomere length of 2·1 $\mu$m, were brought into fatigue by either protocol 1 or protocol 2 as described in the preceding sections. When force had been depressed to a steady level with the respective stimulation protocol, the Perspex chamber with Ringer solution was quickly removed and the fibre was stimulated to produce a 1 s fused tetanus while suspended in air (see Methods). Soon after the onset of force development (see Fig. 8*A*) the fibre was released to shorten against a small load to 1·6 $\mu$m sarcomere length at which point the movement was stopped and the fibre was allowed to develop isometric force. During the plateau of force development at the short length the fibre was rapidly frozen by submersion into a propane–propylene medium that was cooled to liquid nitrogen temperature. Control experiments were performed in which the same procedure of shortening, force development and rapid freezing was carried out during tetanus of non-fatigued fibres, i.e. preparations which had previously been paced to contract at 5 min intervals. Twelve complete experiments of this kind were performed: four control experiments, three experiments based on fatiguing protocol 1 and five experiments using protocol 2. Two or more segments (approximately 1 mm in length and embedded in Epon, see Methods) were randomly selected from each fibre for electron microscopical examination.

Figure 8*B–D* illustrates longitudinal sections of three muscle fibres that were fixed during force development near 1·6 $\mu$m sarcomere length as described above. The non-

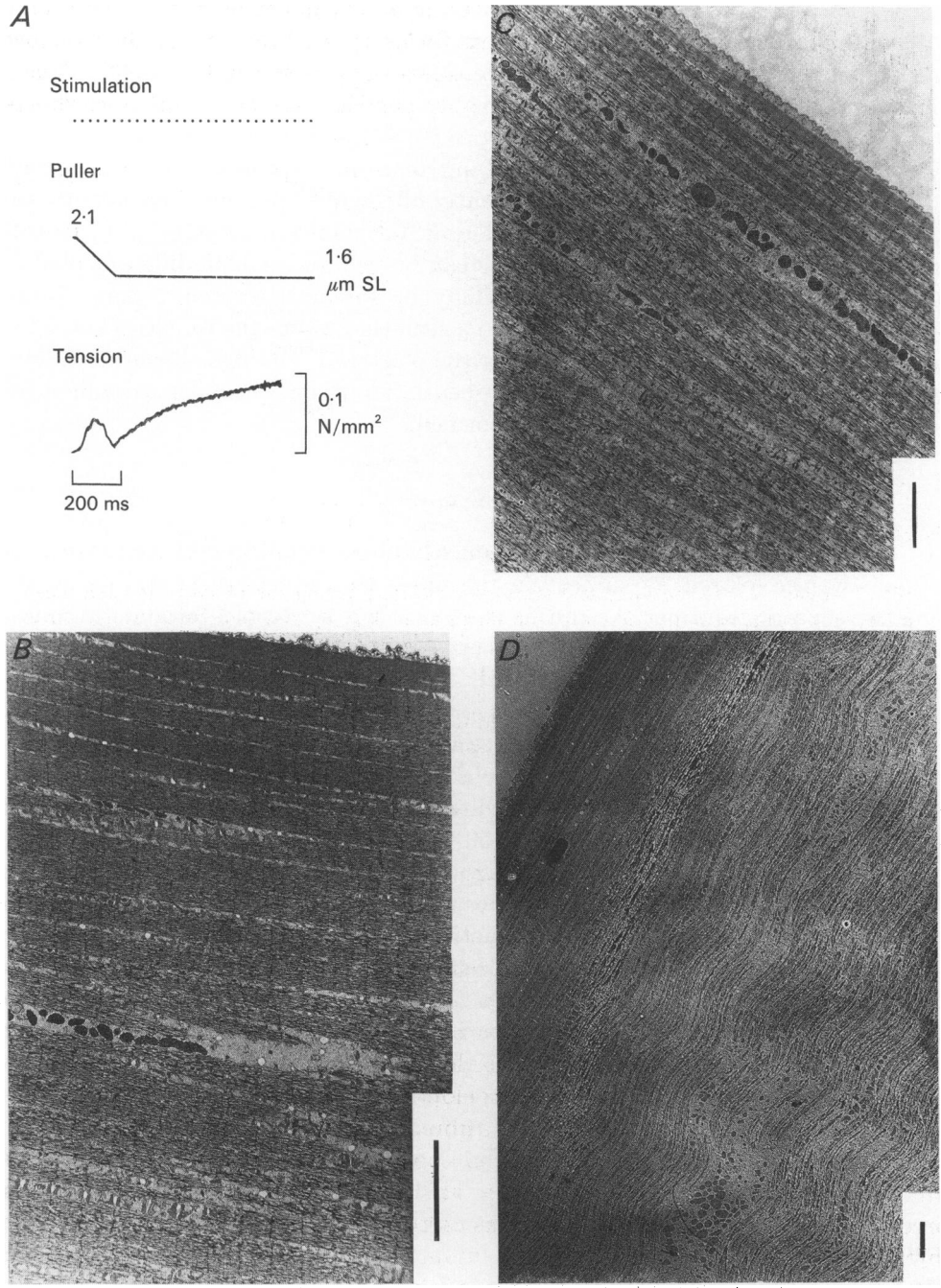


Fig. 8. Longitudinal sections of single muscle fibres fixed by rapid freezing near 1·6 $\mu$m sarcomere length during tetanic contraction. *A*, oscilloscope records illustrating performance of experiment as described in text. Puller signal indicates mean sarcomere length (SL) of muscle fibre. *B*, control (non-fatigued) fibre. *C*, fibre fatigued according to protocol 1. *D*, fibre fatigued according to protocol 2. Note waviness of central myofibrils in *D* indicating failure of the inward spread of activation as further described in text. Scales in *B–D*, 5 $\mu$m.

fatigued fibre (Fig. 8*B*) and the fibre fatigued by stimulation protocol 1 (Fig. 8*C*) both exhibit straight myofibrils throughout the preparation with no sign of waviness that would suggest incomplete sarcomere shortening in any region of the fibre. By contrast, the myofibrillar pattern can be seen to be greatly irregular in the fibre fatigued by stimulation protocol 2 (Fig. 8*D*). Thus, whereas the outer layers of myofibrils have a straight appearance and maintain a parallel orientation after shortening, the myofibrils towards the centre of the fibre become increasingly wavy and, at the same time, less well ordered. This latter point is indicated by the fact that the waviness of the contractile structures can be seen to occur in different planes in neighbouring myofibrils. These results may be taken as evidence that 'fatigue' produced according to protocol 2 leads to a situation where the centre of the muscle fibre becomes incompletely activated by the electrical stimulus. Results similar to those illustrated in Fig. 8*B–D* were obtained in all other specimens examined from the three categories of experiments performed.

## DISCUSSION

In the present study of frog isolated muscle fibres two different levels of muscle 'fatigue' were explored by subjecting the fibres to frequent electrical stimulation using two separate routines. According to stimulation protocol 1 fatigue was induced by reducing the intervals between consecutive tetani from 300 (control) to 15 s. This led to a decrease in amplitude of both twitch and tetanus to 70–80% of the prefatigue values. Stimulation protocol 2 formed a more intense fatiguing programme in that the fibre was stimulated to produce an isometric twitch at 1–2 s intervals thus leaving a minimum of time for recovery between contractions. Using this approach it was possible to reduce the twitch amplitude to 5–10% and the maximum tetanic force (during 1 s stimulation) to 40–50% of the original values. Stimulation programmes similar to protocol 2, ending in a profound reduction of the contractile strength, have been employed in most previous studies of muscle fatigue in isolated preparations (for references, see Introduction).

The present results provide evidence that the two states of 'fatigue' produced by protocols 1 and 2 differ with respect to the cellular mechanisms involved. Thus, whereas fatigue induced by protocol 1 can be accounted for by reduced mechanical performance of the myosin cross-bridges with no significant change in activation of the myofilament system, the more excessive reduction in contractile strength resulting from protocol 2 is largely due to failure of activation of the muscle fibre. These differences concerning the underlying mechanisms of the two fatigue states will be considered in further detail.

### *Moderate fatigue, stimulation protocol 1*

Previous evidence suggests that tetanic force may be reduced to 70–75% of its normal value by fatiguing stimulation, i.e. to the level attained by fatiguing protocol 1, with no apparent change in activation of the contractile system. This is indicated by the finding (Edman & Lou, 1990) that caffeine, added in a twitch potentiating concentration, does not affect the total amplitude of the tetanus in the fatigued state. This probably means that the contractile system is fully activated during tetanus at

the fatigue level considered thus making a further increase in calcium concentration by caffeine ineffectual. The present study provides additional evidence in support of the view that fatigue produced according to protocol 1 is not based on failure of activation of the myofilament system. The results show that the contracture responses to submaximal and supramaximal concentrations of caffeine and to increased potassium concentration are all reduced to the same degree as is the isometric tetanus after development of fatigue. This finding suggests strongly that fatiguing stimulation leads to some change within the muscle fibre that makes the myosin cross-bridges less capable of producing force at any given state of activation of the contractile system.

In confirmation of our previous findings (Edman & Lou, 1990) the present results demonstrate that stiffness is reduced considerably less than is the tetanic force as the muscle fibre goes into fatigue. Thus, for a 25% depression of maximum tetanic force there is merely 9% reduction of fibre stiffness (Fig. 6*B* and Edman & Lou, 1990). This leads to the conclusion that only a minor portion of the force deficit during fatigue is due to fewer attached cross-bridges. The main cause of the force decline after frequent stimulation is therefore reduced force output of the individual cross-bridge.

The altered cross-bridge performance during fatigue further manifests itself as a decrease in maximum speed of shortening (Edman & Mattiazzi, 1981). This would seem to imply that not only is the force-producing capability of the individual cross-bridge diminished in the fatigued state, the maximum speed at which the bridge is able to pass through a cycle of activity is also reduced. Neither of these changes in cross-bridge performance are attributable to failure of activation of the contractile system at the fatigue level considered. The decrease in contractile performance dealt with here may thus be characterized as a true 'myofibrillar fatigue'. The impaired cross-bridge function is likely to be a consequence of the altered metabolic state that arises in the course of muscle fatigue. This point has been discussed in further detail before (Edman & Lou, 1990).

The 'myofibrillar fatigue' outlined above can be presumed to play a significant part during muscular exercise *in vivo* as all the evidence suggests that it arises as a direct consequence of increased activity of the myofibrils. The contractile effects of previous mechanical activity are readily seen in experiments on isolated fibre preparations in which even a very small change of a given stimulation routine results in altered force output during twitch and tetanus (see Edman & Mattiazzi, 1981). There is no reason to believe that the fibre would behave differently in this respect when operating *in situ* in the body.

*Excessive fatigue, stimulation protocol 2*

Stimulation protocol 2 leads to extreme depression of the isometric twitch and to a decrease in tetanic force to less than 50% of the control value. This stimulation programme thus results in a state of exhaustion of the isolated preparation that is reminiscent of extreme muscle fatigue under *in vivo* conditions.

There is reason to believe that the mechanism underlying the initial portion of fatigue induced by protocol 2, i.e. the decline in tetanic force to 70–75% of the control value, is the same as that involved when protocol 1 is used. This is suggested

by the finding that the contractile changes during twitch and tetanus are indistinguishable in the two situations. Furthermore, at the moderate level of fatigue considered, there is no significant tension creep during the tetanus plateau with either of the two stimulation protocols and, most importantly, the force–stiffness relationship is very similar in the two cases. Taken together these findings suggest strongly that the initial decline in contractile performance that occurs in response to protocol 2 does represent the same kind of 'myofibrillar fatigue' that is induced by protocol 1.

The further decline in contractile strength produced by protocol 2, i.e. the decrease in tetanic force below *ca* 70% of the control level, is largely attributable to failure of activation of the muscle fibre. Evidence in support of this view is provided by the finding that the maximal contracture response to caffeine is little affected at this extreme state of 'fatigue' of the muscle fibre (Fig. 5, present paper; Eberstein & Sandow, 1963; Nassar-Gentina *et al.* 1981; Kanaya *et al.* 1983; Lännergren & Westerblad, 1989). Thus, at a point where tetanic force (during 1 s stimulation) has been depressed to 40–50% of its original value the caffeine contracture is merely reduced by a few per cent, i.e. to 97–98% of its control ('rested-state') value (Fig. 5). The high-frequency stimulation of protocol 2 apparently leads to some failure of the excitation–contraction coupling that is circumvented when the fibre is activated by caffeine which is generally thought to induce calcium release by acting directly upon the intracellular storage site of activator calcium.

Our results show (Fig. 8) that high-frequency stimulation eventually leads to impairment of the inward spread of activation into the muscle fibre, and this effect is likely to account for a major part of the force decline during twitch and tetanus at an advanced state of fatigue of the isolated muscle preparation. With the technique used it was possible to demonstrate that myofibrils in the centre of the fibre were unable to shorten actively below slack length during tetanus in the fatigued state indicating that they were not properly activated by the electrical stimulus. Failure of the inward spread of activation after frequent tetanization has previously been reported by Gonzalez-Serratos *et al.* (1981) and Garcia *et al.* (1991) and was thought by these authors to be the principal mechanism of muscle fatigue. The present results (Fig. 8) clearly show, however, that failure of activation does not arise unless the intervals between consecutive contractions are made exceedingly short (1–3 s). Frequent stimulation is likely to cause accumulation of potassium in the T-tubules when too short a time is provided to restore the potassium concentration of the tubular fluid between contractions (e.g. Hnik *et al.* 1976). The increased potassium concentration will lead to depolarization of the tubular membrane and this will impair the inward spread of the electrical impulse along the transverse tubuli. In the extreme case the centre of the fibre will stay totally inactive while only the outer layers will be within reach of the action potential and thus be accessible for activation. Frequent stimulation will also, probably, reduce the sodium concentration in the transverse tubules (Juel, 1986), but this change is unlikely to seriously affect the regenerative spread of the electrical impulse (Juel, 1988). It is noteworthy that there was no sign of deficient activation during fatigue induced by protocol 1. The 15 s rest period between tetani during this stimulation programme was apparently sufficient to keep the potassium concentration in the T-tubules at a

low enough level to enable the action potential to propagate all the way into the centre of the fibre.

Incomplete inward spread of activation probably underlies the marked 'tension creep' and the drop in twitch–tetanus ratio that occur after an intense period of fatiguing stimulation (Fig. 5*B*). The impaired inward spread of the action potential will lead to decremental activation towards the centre of the fibre as explained above. The amount of calcium released by a single stimulus is likely to be much reduced under these conditions and the twitch response correspondingly depressed. By contrast, the repetitive release of calcium during tetanic stimulation can be presumed to steadily raise the myoplasmic calcium concentration and thus cause a slow climb of force during the stimulation volley. This accords with the observation that tension continues to rise for 2–3 s, or even longer, during tetanic stimulation after the fibre has been fatigued according to protocol 2 (Fig. 5*B*). The concomitant increase in fibre stiffness during tension creep (Fig. 7) provides evidence that new cross-bridges are indeed steadily recruited during the creep phase. The fact that force, even after completion of tension creep, was lower than that attained in fatigue with protocol 1 supports the view that the innermost part of the fibre eventually becomes inaccessible for activation by the electrical impulse and therefore does not contribute to force production during tetanic stimulation.

As is evident from the foregoing, fatiguing protocol 2 eventually leads to depression of the isometric twitch and tetanus that is largely attributable to failure of propagation of the action potential along the T-tubuli. Somewhat ironically a situation may therefore arise in which a substantial part of the fibre remains passive, and thus resting, while fatiguing stimulation goes on. It is still unclear if fatigue according to protocol 2, at least in a more developed form, is physiologically relevant as it has not yet been demonstrated that muscle fibres *in situ* in the body may ever be subjected to a stimulation regimen intense enough to impair the inward spread of activation. There is evidence suggesting (Marsden, Meadows & Merton, 1971; Bigland-Ritchie, Dawson, Johansson & Lippold, 1986; Garland & McComas, 1990) that the motor input to body muscles *in vivo* is modulated during fatigue in a way that serves to protect the muscles from excessive stimulation.

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